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Intracellular pH regulation in isolated trout gill mitochondrion-rich (MR) cell subtypes: Evidence for Na^+/H^+ activity

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ABSTRACT

We have studied intracellular pH (pH_i) recovery in isolated trout gill mitochondrion-rich (MR) cells following acidification by the NH_4Cl pre-pulse technique. Within a mixed MR cell population, one cell type displayed Na^+-independent pH_i recovery while the other cell type lacked a Na^+-independent pH_i recovery. Cells displaying Na^+ independent recovery exhibited a significantly higher buffering capacity compared to cells lacking Na^+-independent pH_i recovery. Cells displaying Na^+ independent recovery were identified as PNA^+ (peanut lectin agluttinin binding) MR cells while those unable to recover were identified as PNA^- (non-peanut lectin agluttinin binding) MR cells. Therefore, recovery from acidification in the absence of Na^+ provides a direct functional marker for PNA^+ and PNA^- MR cells. Re-addition of Na^+ to acidified cells resulted in a transient pH_i recovery in both cell types. This event was abolished by amiloride (500 μM) but it was insensitive to phenamil (50 μM). The phorbol ester PMA (1 μM) potentiated the Na^+ induced pH_i recovery suggesting that activation by PKC is required for continuous Na^+/H^+ exchanger activity in trout gill MR cells. This study is the first functional description of pH_i recovery in lectin-identified trout gill MR cells and provides insight into a putative cellular signaling mechanism that may control pH_i regulation in the gill epithelium.

1. Introduction

Maintenance of constant intracellular pH (pH_i) is essential for proper cell function and survival (Roos and Boron, 1981). Not surprisingly, efficient transporters exist on cellular membranes to ensure regulation of pH_i within narrow limits. The ubiquitous "housekeeping" Na^+/H^+ exchanger (NHE1) is the primary protein responsible for maintaining pH_i across a broad range of cell types (for review see (Orlowski and Grinstein, 2004).

Mitochondrion-rich (MR) cells in the fish gill epithelium have been extensively studied due to their importance in maintaining blood acid–base and ion levels. However, information for pH_i regulation is completely unknown and the proposed mechanisms have to be extrapolated from studies focusing on systemic pH and ion regulation. MR cells express a variety of acid–base relevant proteins including carbonic anhydrase, V-type H^+-ATPase (VHA), Na^+/HCO_3^- co-transporters (NBC), Cl^-/HCO_3^- exchangers (CBE), and the aforementioned NHE (for review see (Evans et al., 2005; Perry and Gilmour, 2006; Tresguerres et al., 2006; Hwang and Lee, 2007; Evans, 2008; Parks et al., 2008). In gills from seawater fishes, current models predict that some of these proteins are differentially expressed in at least two distinct cell subtypes, each of them achieving acid or base secretion (Piermarini and Evans, 2001; Hawkings et al., 2004; Tresguerres et al., 2005; Perry and Gilmour, 2006). This is similar to the gills of freshwater trout where two distinct MR cell subtypes were identified based on their ability to bind peanut lectin agluttinin. The terms PNA^+ and PNA^- MR cells (Goss et al., 2001) were coined to coincide with the mammalian literature where functionally and morphologically analogous cell types in the cortical collecting duct of the rabbit kidney were found with differential PNA staining (Lehir et al., 1982). Further studies demonstrated the importance of VHA (Galvez et al., 2002), Na^+ channels (Reid et al., 2003; Parks et al., 2007), and NBCs (Parks et al., 2007) in the PNA^- MR cell fraction while CBEs and Cl^--dependent NHEs were shown to be present in both MR cell subtypes (Parks et al., 2009). The combined evidence suggests that PNA^- MR cells are responsible for Na^+ uptake and H^+ secretion using apical sodium channels coupled to VHA and coordinated with basolateral NBC and Na^+/K^+-ATPase (NKA), while PNA^+ MR cells perform Cl^- uptake and HCO_3^- secretion through transporters of unknown molecular identity (Perry and Gilmour, 2006; Tresguerres et al., 2006; Hwang and Lee, 2007; Parks et al., 2008, 2009).

Only a few studies have directly monitored the pH_i of gill cells and these found functional evidence for NHE activity in cultured gill

Abbreviations: CBE, (Cl^-/HCO_3^- exchangers); MR, (mitochondrion-rich); NKA, (Na^+/K^+-ATPase); NBC, (Na^+/HCO_3^- co-transporters); NHE, (Na^+/H^+ Exchanger); pH_i, (intracellular pH); PKA, (protein kinase A); PKC, (protein kinase C); PNA^+, (peanut lectin agluttinin binding); PNA^-, (non-peanut lectin agluttinin binding); VHA, (V-type H^+-ATPase); zNHE1, (zebrafish Na^+/H^+ Exchanger isoform 1).

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pavement (PV) cells of goldfish (Sandbichler and Pelster, 2004) and trout (Part and Wood, 1996; Wood and Part, 2000). However, functional evidence for NHE activity and pH_i regulation in gill MR cells is absent. The red blood cell β-NHE was the first transporter to be cloned from trout (Borgese et al., 1992). That seminal paper demonstrated that the β-NHE could be activated by cAMP *via* PKA or PKC pathways independent from each other (Borgese et al., 1992). Studies on sculpin and killifish lead to the hypothesis that β-NHE performed the function of NHE1 at the gill (Claiborne et al., 1999). Hwang's laboratory (Yan et al., 2007) has recently cloned all of the known NHE isoforms from zebrafish. Phylogenetic analysis grouped zebrafish NHE1 (zNHE1) closely with trout β-NHE and zNHE1 was strongly expressed in erythrocytes contrary to mammalian expression patterns (Yan et al., 2007). Although zNHE1 was detectable in the gill *via* RT-PCR, no expression was noted in gill cells *via in situ* hybridization. Recently, NHE2 and NHE3 genes have been cloned from rainbow trout and found to be expressed in gill and kidney (Ivanis et al., 2008a,b), however, a trout homolog to NHE1 has not yet been established. Direct evidence for NHE1-like activity in gill MR cells along with characterizations of the cellular signaling mechanisms controlling ion and acid–base transport are lacking and remain an area of future research. Our goal in this study was to provide evidence for the mechanisms responsible for pH_i recovery in identified trout gill MR cell subtypes along with characterization of cellular signaling mechanisms involved in pH_i-related ion transport. We provide physiological markers for MR cell subtypes, buffering capacity differences between subtypes, and a Na^+ dependent pH_i recovery mechanism that requires PKC for activation. This is the first study to report a potential mechanism to control pH_i regulation of MR cells in the gill epithelium.

2. Materials and methods

2.1. Experimental animals

Adult rainbow trout (*Oncorhyncus mykiss*) used in this study were reared as described previously (Parks et al., 2007; Parks et al., 2009). Briefly, fish were maintained in flow through 450-liter fibreglass tanks filled with continuously aerated and dechlorinated City of Edmonton tap water (hardness of 1.6 mmol/L as $CaCO_3$, total alkalinity of 120 mg/L, pH 8.2 (~0.5 mmol/L NaCl). Water temperature in the tanks was maintained permanently at 15 °C, and the photoperiod mimicked the natural pattern found in Edmonton, Alberta, Canada. Fish were fed once per day with dry commercial trout pellets. Use of these animals followed Canadian Council on Animal Care and approved Biological Sciences Animal Care Committee protocol #215507.

2.2. Isolation of MR cells

Isolation of MR cells from the trout gill epithelium followed the techniques developed by Goss et al. (2001). For a detailed description of the procedure that was utilized please see our previous papers (Parks et al., 2007, 2009) as the same technique was used in this current study. For identification of MR cell subtypes following Percoll separation, MR cells were incubated with 40 μg/mL PNA-biotin for 30 min, washed two times with PBS, then incubated for 30 min with streptavidin-conjugated Alexa fluor 594 (Molecular Probes). Prior to pH_i imaging, cells were identified as either PNA^+ and PNA^- cells based on the presence of peripheral staining with Alexa fluor 594 using a Texas Red filter set (560 nm excitation, 630 nm emission).

2.3. Intracellular pH (pH_i) imaging

Intracellular pH (pH_i) imaging followed procedures described previously (Parks et al., 2007, 2009). Fluorescent imaging of MR cells loaded with the pH sensitive dye BCECF-AM (Molecular Probes—5 μM) was performed at a room temperature of 18 °C in a 70-μL imaging chamber (RC-20H; Warner Instrument). Differential interference contrast microscopy (Nikon Eclipse TM-300) and fluorescence imaging (TE-FM epifluorescence attachment) were performed with an inverted microscope. The microscope was fitted with a xenon arc lamp (Lambda LS; Sutter Instruments, Novato, CA, USA) to enable excitation of the BCECF-AM-loaded cells at wavelengths of 495 and 440 nm. Images at both 440 and 495 nm were captured digitally on a mono 12-bit charge-coupled device camera (Retiga EXi; Burnaby, BC, Canada) every 5 s during the various perfusion experiments. Northern Eclipse version 6 software (Mississauga, ON, Canada) was used to compile the 495-to-440 nm ratios as an indication of the pH_i levels.

2.4. Perfusion protocol

Solutions were added to the perfusion chamber using a six-input manifold (Mp-6; Warner Instrument) attached to gravity-feed, 60-mL syringes in syringe holder blocks equipped with pinch valves (VE-6; Warner Instrument) and controlled by VC-6 valve controllers (Warner Instrument). The perfusion rate was adjusted to ~0.5 mL/min. Cells were alkalinised and acidified using the NH_4Cl (20 mM) pre-pulse technique (Boron and Deweer, 1976) in Na^+ free medium (in mM: 125 *N*-methyl-D-glucamine-Cl, 2.5 $C_5H_{14}NO{\cdot}HCO_3^-$, 5 $CaCl_2$, 1 $MgCl_2$, 4 KCl, 2 glucose, 15 HEPES; pH 7.8, 290 mOsm). Recovery of pH_i was then observed in both Na^+ free and Na^+-containing solutions (in mM: 125 NaCl, 5 $CaCl_2$, 1 $MgCl_2$, 4 KCl, 2.5 $NaHCO_3$, 2 glucose, 15 HEPES; pH 7.8, 290 mOsm). Pharmacological profiling was done using amiloride (500 μM), phenamil (50 μM) and PMA (1 μM). All of the solutions used were bubbled continuously with a gas mixture of 0.3% CO_2 balanced with O_2 for most experiments. The high-K^+ (in mM: 120 potassium gluconate, 20 KCl, 2 $MgCl_2$, 20 HEPES) nigericin (5 μM) four step calibration of pH_i was used at the end of each experiment as described previously (Parks et al., 2007). Similar experiments on labelled PNA^+ and PNA^- MR cells were also performed with Na^+ free (in mM: 130 *N*-methyl-D-glucamine-Cl, 1.5 $CaCl_2$, 1 $MgCl_2$, 5 KCl, 5 glucose, 1 HEPES; pH 7.8, 290 mOsm) and Na^+ containing solutions (in mM: 130 NaCl, 1.5 $CaCl_2$, 1 $MgCl_2$, 5 KCl, 5 glucose, 1 HEPES; pH 7.8, 290 mOsm).

2.5. Calculation of buffering capacity (β)

Calculation of intracellular buffering capacity was estimated following the calculations described by Graber et al., (1991). The buffering capacity (β, mM/pH unit) was calculated according to the following basic formula:

$$\beta = \Delta NH_{4\ \mathrm{in}}^{+} / \Delta pH_i.$$

This estimate of the acid load assumes that all NH_4^+ exits the cell as NH_3 giving up an H^+ in the process as described previously (Graber et al., 1991). Calculations of NH_4^+ were done assuming a pK of 9.03 and equilibration of intra- and extracellular NH_3 at an extracellular pH of 7.80. Mathematical calculations followed the exact description by Graber et al. (1991). We obtained our values for buffering capacity in Na^+ free experiments to limit the confounding variable of a Na^+ dependent acid extruding system interfering with our calculations.

2.6. Data analysis

Fluorescence ratios generated from experiments with BCECF were converted to pH_i values using Microsoft excel. For pharmacological profiling, initial rates of pH_i change (slope) were monitored over the linear portion of the response (either 30 or 60 s intervals) following manipulation to give a control and experimental rate of pH change (pH units/min) for comparison *via* Student's *t*-test or repeated

measures one-way ANOVA with Tukey post-hoc test. Summary data are listed as the mean ± the standard error. Final figure composition and statistical analysis was performed using GraphPad Prism version 3.0 software (San Diego, CA, USA). Statistically significant differences ($p<0.05$) are indicated on the bar graphs by either an * or a letter. All analyses are from MR cells obtained from a minimum of 3 different fish and 5 separate experiments. Unless otherwise mentioned, the reagents used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA).

3. Results

3.1. Na^+ free recovery following NH_4Cl induced acidification and buffering capacity

Following intracellular acidification by NH_4Cl only one sub-set of MR cells were able to demonstrate pH_i recovery in Na^+ free conditions (Fig. 1a). The ability to recover pH_i in Na^+ free conditions establishes a functional separation of MR cells as described previously (Parks et al., 2007). These behaviours corresponded to a difference in buffering capacity (β) between the two MR cell subtypes (Fig. 1b) with the Na^+ free recovering cells having a significantly higher buffering capacity ($\beta = 18.71 \pm 5.19$ mM/pH unit) than the non-Na^+ free recovering cells ($\beta = 2.07 \pm 0.46$ mM/pH unit).

3.2. PNA^+ and PNA^- MR cells correspond to functional behaviours

The rate of pH_i recovery following NH_4Cl-induced acidification was also measured in identified PNA^+ and PNA^- MR cells. Recovery from acidosis in Na^+ free conditions occurred in the PNA^+ MR cell fraction only (Fig. 2a). PNA^+ cells also had a higher resting pH_i and higher buffering capacity (β~2× higher, Fig. 2b) than PNA^- cells. This matches the characteristics of the Na^+ free recovering cells described above and previously (Parks et al., 2007). Therefore, recovery from NH_4Cl induced acidification in the absence of Na^+ can be used as a marker for PNA^+ MR cells.

3.3. Effect of Na^+-addition on pH_i recovery following an acid load

Introduction of Na^+-containing buffer following acidification with a NH_4Cl pre-pulse resulted in a robust surge in pH_i recovery in both cell types, which was followed by a rapid re-acidification (Fig. 3). This transient recovery-acidification event was also observed following a second acidification of the same cells (compare dashed boxes in Fig. 3). The repeatability of this event allowed us to examine the effect of pharmacological agents on this transient Na^+ dependent pH_i recovery.

3.4. Effect of amiloride on Na^+ dependent pH_i recovery

Amiloride is a general inhibitor of Na^+/H^+ exchangers as well as Na^+-channels (Kleyman and Cragoe, 1988). The transient pH_i

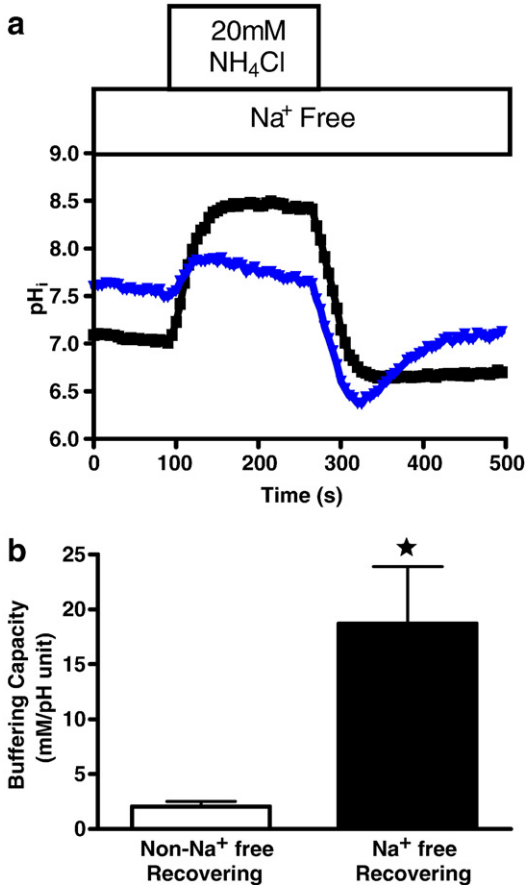

Fig. 1. Na^+ free recovery from acidosis correlates to higher cellular buffering capacity. *a* Sample trace showing a cell that recovers from NH_4Cl induced acidosis in Na^+ free medium (blue trace) compared to one that does not (black trace). *b* Na^+ free recovering cells have a significantly higher buffering capacity (18.71 ± 5.19 mM/pH unit, $n=33$) compared to non-Na^+ free recovering cells (2.07 ± 0.46 mM/pH unit , $n=37$). $p<0.05$ unpaired non-parametric Mann Whitney *t*-test.

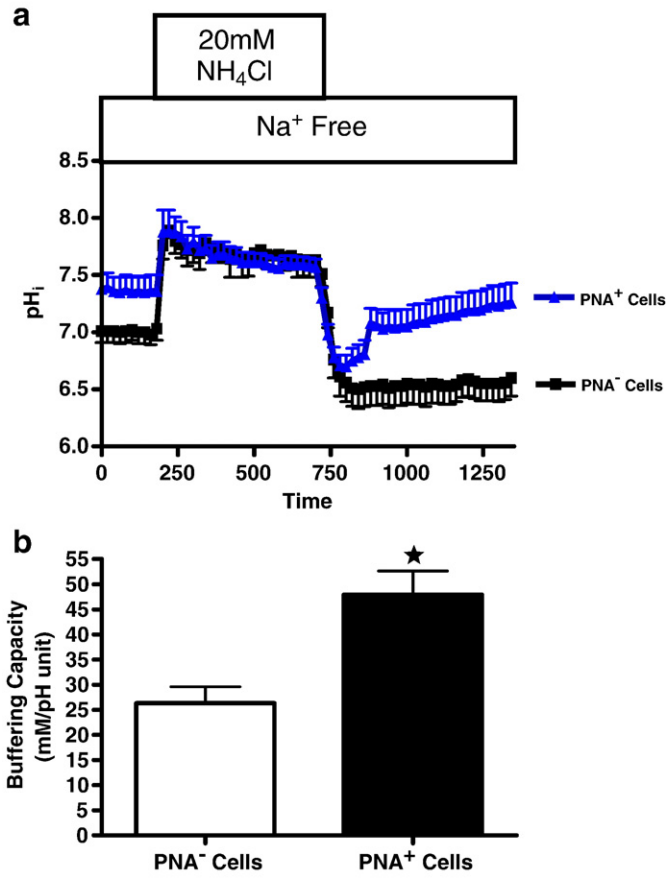

Fig. 2. PNA^+ and PNA^- MR cell responses to NH_4Cl induced acidosis. *a* PNA^+ MR cells (blue trace $n=16$) demonstrate Na^+ free recovery following NH_4Cl induced acidosis, while PNA^- MR cells do not (black trace $n=6$). *b* Buffering capacity of PNA^+ and PNA^- MR cells. $p<0.05$ unpaired non-parametric Mann Whitney *t*-test.

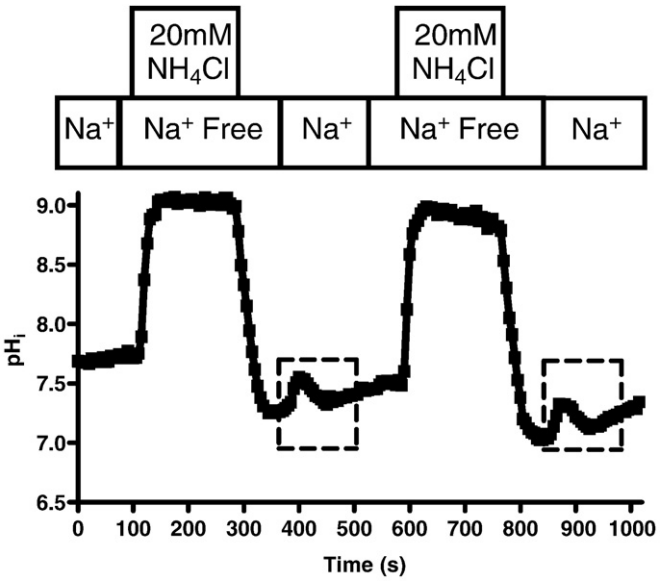

Fig. 3. Representative trace demonstrating the effect of Na^+ addition following a 20 mM NH_4Cl pre-pulse induced acidification. Dashed box highlights a transient Na^+ dependent alkalinization and re-acidification. Cells were subjected to two consecutive acidification events and displayed the same behavior following initial and secondary acidifications (compare the two dashed boxes, $n=7$).

recovery induced by Na^+ addition ($\Delta pH_i/\Delta t$ of 0.66 ± 0.08 pH units/min) was effectively removed by 500 μM amiloride ($\Delta pH_i/\Delta t$ of 0.07 ± 0.04 pH units/min, Fig. 4a and b). However, phenamil (50 μM) a known pharmacological blocking agent specific for Na^+ channels but with no known inhibition of NHEs did not inhibit the Na^+ induced recovery (Fig. 4c). Since the same concentration of phenamil is effective at blocking a putative apical Na^+ channel (Parks et al., 2007), we conclude that the transient intracellular alkalinization is due to an amiloride-sensitive NHE.

3.5. Effect of the phorbol ester PMA on Na^+ induced pH_i recovery

Protein kinase C (PKC) has been linked to NHE activation in the trout red blood cell β-NHE (Borgese et al., 1992; Motais et al., 1992; Malapert et al., 1997) and this led us to test the involvement of PKC on the Na^+ dependent pH_i recovery mechanisms found in trout MR cells. Following a control acidification/recovery, cells were exposed to a second acidification and recovery but with the addition of 1 μM PMA. This resulted in a sustained pH_i recovery (no re-acidification event) in both the Na^+-dependent and independent recovering cell populations (Figs. 5a and 6a). In PNA- MR cells (those displaying no recovery in Na^+ free conditions-Fig. 2), PMA resulted in a sustained Na^+-induced pH_i recovery ($\Delta pH_i/\Delta t$ of 0.38 ± 0.06 pH units/min) that was not significantly different from the initial transient Na^+-induced alkalinization ($\Delta pH_i/\Delta t$ of 0.42 ± 0.06 pH units/min, Fig. 5a and b). Cells demonstrating Na^+-independent pH_i recovery (PNA^+ MR cells-Fig. 2) had an initial pH_i recovery rate of 0.49 ± 0.11 pH units/min in Na^+ free medium. Addition of Na^+ temporarily increased the rate significantly ($\Delta pH_i/\Delta t = 0.80 \pm 0.05$ pH units/min) but the rate later returned to a value that was not significantly different from the original Na^+ free conditions ($\Delta pH_i/\Delta t$ 0.36 ± 0.04 pH units/min) (Fig. 6a and b). PMA did not affect the Na^+ independent recovery ($\Delta pH_i/\Delta t$ 0.46 ± 0.05 pH units/min), but it maintained the elevated rate of pH_i recovery observed during the early phase of recovery upon Na^+ addition (0.90 ± 0.12 pH units/min) (Fig. 6a and b). The concentration of PMA that we used was similar to what was demonstrated to be an effective dose in trout kidney cells (Lacroix and Hontel, 2001) and hepatocytes (Reader et al., 1999).

4. Discussion

4.1. Functional separation of gill MR cell subtypes

Pisam et al. first described two types of MR cells (termed chloride cells at the time) based on morphological characteristics (Pisam et al., 1987, 1993). Development of a percoll separation technique to isolate MR cells from Japanese eel was a key finding to allow the functional characterization of these cells (Wong et al., 1999a,b). Goss et al. then reported the identification of MR cells subtypes at the freshwater trout gill based on peanut lectin agglutinin (PNA) binding (Goss et al., 2001; Galvez et al., 2002) eventually leading to a method to functionally separate and characterize the isolated MR cell subtypes (Galvez et al., 2002; Reid et al., 2003; Hawkings et al., 2004).

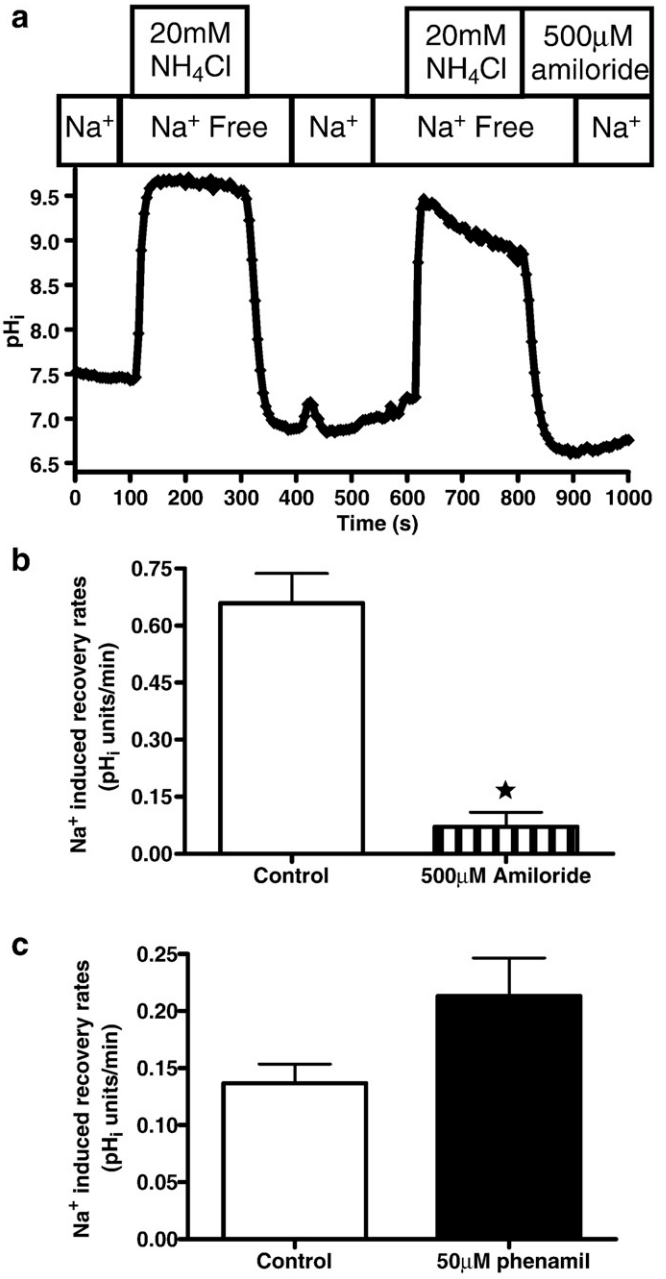

Fig. 4. The effect of 500 μM amiloride on the transient Na^+ dependent pH_i recovery. *a* Representative trace demonstrating abolishment of the transient Na^+-dependent pH_i recovery with the addition of amiloride (500 μM). *b* Summary statistics for part a ($p<0.05$, paired *t*-test, $n=30$ cells). *c* Summary statistics for the effect of 50 μM phenamil on the transient Na^+ dependent pH_i recovery ($p>0.05$, paired *t*-test, $n=23$ cells).

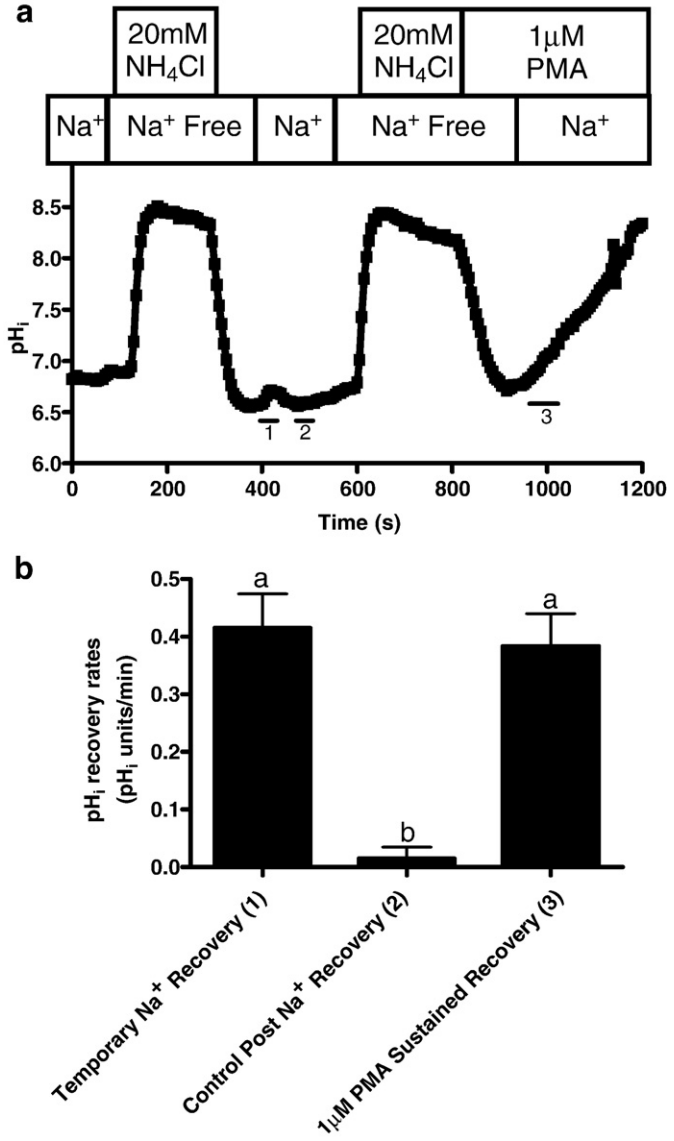

Fig. 5. The effect of 1 μM PMA on Na^+-induced pH_i recovery from acidification following exposure to 20 mM NH_4Cl in PNA^- cells. *a* Na^+-dependent pH_i recovery is sustained in the presence of 1 μM PMA. Lines and corresponding numbers under the sample trace correspond to regions of pH_i recovery analysed in summary statistics. *b* Summary statistics for specific regions (1–3) of the traces demonstrated in part a. Significant differences between groups are indicated by letters. $p<0.05$, repeated measures one-way ANOVA, Tukey post-hoc test, $n=16$.

However, this procedure substantially extends the time required for cell isolation and results in a lower cell yield, thus limiting the number and type of experiments that can be performed.

Previously we reported the ability of one subtype of MR cells (within a mixed MR cell population) to recover pH_i in the absence of Na^+ (Parks et al., 2007). The NH_4Cl experiments performed in that study were used to demonstrate cell viability during pH_i experiments as it was the first extensive use of the technique involving gill MR cells. However, we went on to describe a model for transepithelial Na^+ uptake in that study and did not pursue the mechanisms responsible for pH_i recovery following NH_4Cl induced acidification. Our results from this study aimed to provide insight into these mechanisms of pH_i recovery and importantly, are the first to provide a direct functional linkage to specifically identified PNA^+ and PNA^- MR cells. Our data showing differential pH_i recovery in Na^+ free conditions following acidification in PNA^- cells and PNA^+ MR cells provide additional evidence for functional differences in isolated trout gill MR cell subtypes. Furthermore, pH_i recovery can be used as yet another way to identify MR cell subtypes within a mixed population of MR cells, because it correlates directly to the behaviours found in separated PNA^+ and PNA^- MR cells respectively.

MR cell subtypes have been found in most fish species investigated so far, using lectins or specific antibodies. These studies revealed important species-specific differences, which demonstrate the difficulties of finding a universal marker for fish MR cell subtypes. For example, zebrafish has at least 2 MR cell subtypes based on co-localization of concanavalin A with VHA and not NKA (Lin et al., 2006; Esaki et al., 2007). These studies in zebrafish have also demonstrated the functional importance of VHA and NHE3 in acid secretion and Na^+ uptake from freshwater in identified ionocytes (a more generic term for MR cells in fish) (Lin et al., 2006; Esaki et al., 2007; Horng et al., 2007; Yan et al., 2007). Interestingly however, concanavalin A co-localizes with NKA in MR cells from tilapia yolk sac membrane which is the exact opposite of the trend in zebrafish (Lin and Hwang, 2004). Moreover, an excellent immunohistochemical analysis suggests as

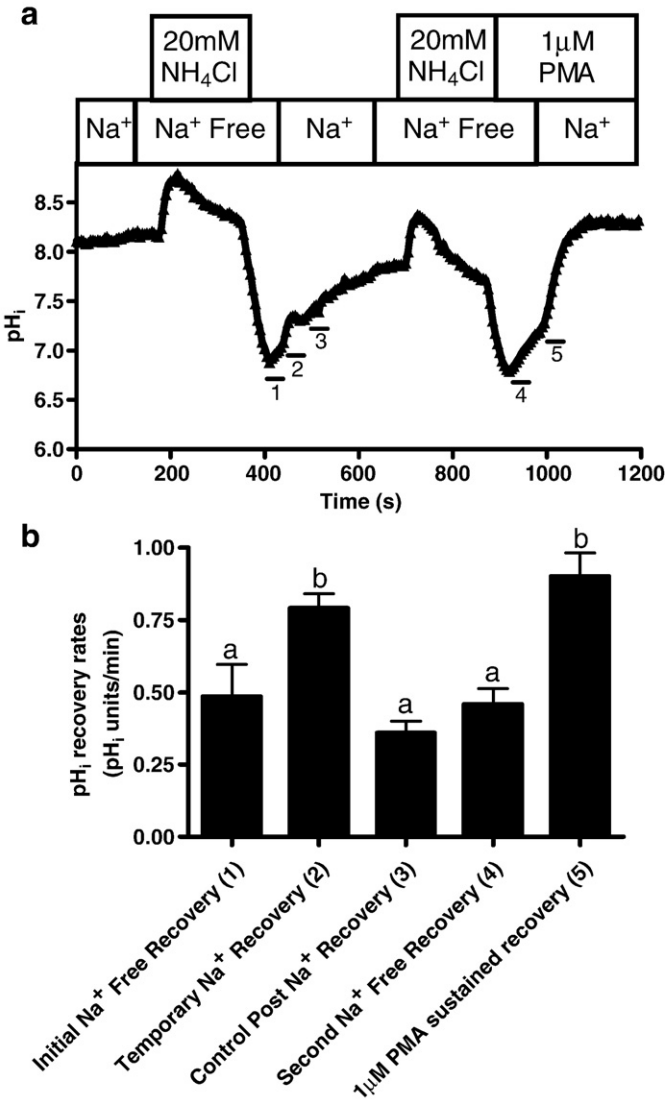

Fig. 6. *a* The effect of 1 μM PMA on Na^+-induced pH_i recovery from acidification following exposure to 20 mM NH_4Cl in PNA^+ cells. Na^+ addition resulted in an additive pH_i recovery which required PMA to be sustained. Lines and corresponding numbers under the sample trace correspond to regions of pH_i recovery analysed in summary statistics. *b* Summary statistics for specific regions (1–5) of the traces demonstrated in part a. Significant differences between groups are indicated by letters. $p<0.05$, repeated measures one-way ANOVA, Tukey post-hoc test, $n=10$.

many as four different MR cell subtypes exist at the gill of tilapia (Hiroi et al., 2008). Our identification method based on the ability to recover pH_i from acidification might prove helpful for finding functional similarities between MR cell subtypes from different species.

Recently, we have provided evidence for the presence of a Cl^-/HCO_3^- exchanger (CBE) in both functionally identified MR cell subtypes (Parks et al., 2009). Consequently, it is reasonable to assume that both MR cell subtypes would be capable of demonstrating a Na^+ free recovery pattern. However, our present study and previous reports (Parks et al., 2007) indicate that only one MR cell subtype (PNA^+ cells) uses the CBE for recovery from acidosis. One possibility is that the CBE in the Na^+ free recovering cells (PNA^+ cells) is more active under our experimental conditions and/or present in greater levels on the membrane due to their attributed *in vivo* role for transepithelial Cl^- uptake (Evans et al., 2005; Tresguerres et al., 2006; Evans, 2008; Parks et al., 2009) whereas the CBE present in PNA^- MR cells (non-Na^+ free recovering cells) is not activated sufficiently by acidosis to achieve pH_i recovery.

4.2. Correlation of MR cell subtypes with cellular buffering capacity

Previous estimates of buffering capacity (β) in HEPES buffered medium for the trout gill epithelia were 13.4 and 13.7 Slykes (mM/pH unit) in cultured PV cells and gill homogenates respectively (Wood and Lemoigne, 1991; Part and Wood, 1996). These fall within the values found in this study for Na^+ free recovering cells (18.71 ± 5.19 mM/pH unit) but are much greater than that found in the non-Na^+ free recovering cells (2.07 ± 0.46 mM/pH unit). This analysis was consistent with that observed in experiments involving identified PNA^+ and PNA^- MR cells where PNA^+ cells exhibited a significantly higher buffering capacity compared to PNA^- cells. Relative differences in buffering capacity may be related to higher levels of intracellular HCO_3^- present in PNA^+ cells versus PNA^- cells. Higher intracellular HCO_3^- in PNA^+ cells may well be utilized to enhance Cl^- uptake, whereas lower HCO_3^- in PNA^- cells could result from the extrusion of HCO_3^- across the basolateral membrane as suggested previously (Parks et al., 2007). Higher HCO_3^- levels in these cells would also explain the higher resting pH_i noted previously in Na^+ free recovering cells compared to non-Na^+ free recovering cells (Parks et al., 2007).

4.3. NHE1-like activity in MR cells pH_i recovery: a role for PKC

Amiloride sensitivity and lack of inhibition by phenamil establish that the alkalinizing effect of Na^+ introduction is dependent on an NHE and not a Na^+ channel linked to a V-H^+-ATPase. We have recently demonstrated the presence of a Cl^- dependent NHE (Cl-NHE) in both trout MR cell subtypes (Parks et al., 2009). However, the Cl-NHE mechanism was insensitive to amiloride (Parks et al., 2009), unlike our current results showing complete abolition by amiloride. To date, three trout NHEs have been molecularly identified: NHE2, NHE3a and NHE3b (also termed SLC9A2 and SLC9A3a and b, respectively) (Ivanis et al., 2008b). Interestingly, the housekeeping NHE-1 homologues have not been cloned for trout. In the trout gill, NHE3 is preferentially located in the PNA^+ MR cells (Ivanis et al., 2008b). However, the subcellular localization does not appear especially apical, and therefore in the role of NHE-3 in PNA^+ MR cells is unclear. In our study, re-addition of Na^+ caused a transient and amiloride-sensitive recovery of pH_i in both cell types. Since PNA^- MR cells lack NHE2 and 3 (Ivanis et al., 2008b), the behaviours noted in our study are likely due to another unidentified NHE isoform. Furthermore, in our system the continuous Na^+/H^+ exchange is dependent on activation by PKC. Activation by PKC matches with the established role for PKC in stimulating trout RBC β-NHE (Borgese et al., 1992; Malapert et al., 1997) and is also consistent with the role of PKC in maintaining the activity of mammalian non RBC NHE1 isoforms (Magro et al., 2005). In contrast, the Na^+ absorbing mammalian isoform NHE3 is inhibited by PKC (Donowitz et al., 2000). Considering all of these factors, we propose that the Na^+ dependent, PMA potentiated pH_i recovery noted in our study is mediated by a NHE1-like protein. However, a definitive conclusion depends on the cloning and pharmacological profiling of the trout NHE-1 like isoform. Furthermore, an extensive molecular and pharmacological profiling will be required to determine which PKC isoforms are involved in pH_i regulatory mechanisms of gill MR cells.

One alternative hypothesis could be that under control conditions, Na^+ introduction first activates an NHE to alkalinize the cell and subsequently activates a net acid transport (H^+ import or HCO_3^- export) that masks the NHE mediated alkalinization. When PMA is present, this masking mechanism would be inhibited resulting in the sustained alkalinization noted. However, the primary candidate for this would be the NBC mediated HCO_3^- extrusion mechanism as observed previously for PNA^- cells only (Parks et al., 2007). Furthermore, PKC activation has been shown to increase both the kidney and intestinal NBC activity (Ruiz et al., 1997; Bachmann et al., 2006) and this response is the exact opposite to that required for our noted responses. Since we demonstrate that PMA activation of recovery is found in both MR cell types, this suggests stimulation of NHE and not inhibition of NBC.

In summary, we have provided additional information for the physiological differences of trout gill MR cell subtypes and established a physiology-based method to identify these subtypes (Na^+ dependent pH_i recovery). These data are also the first evidence for mechanisms that control pH_i regulation in isolated gill MR cells and suggest that pH_i imaging is an appropriate method to determine intracellular signalling pathways that regulate acid–base transport in gill cells.

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